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Multiple-Peptidase Mutants of *Lactococcus lactis* Are Severely Impaired in Their Ability To Grow in Milk

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To examine the contribution of peptidases to the growth of *Lactococcus lactis* in milk, 16 single- and multiple-deletion mutants were constructed. In successive rounds of chromosomal gene replacement mutagenesis, up to all five of the following peptidase genes were inactivated (fivefold mutant): *pepX*, *pepO*, *pepT*, *pepC*, and *pepN*. Multiple mutations led to slower growth rates in milk, the general trend being that growth rates decreased when more peptidases were inactivated. The fivefold mutant grew more than 10 times more slowly in milk than the wild-type strain. In one of the fourfold mutants and in the fivefold mutant, the intracellular pools of amino acids were lower than those of the wild type, whereas peptides had accumulated inside the cell. No significant differences in the activities of the cell envelope-associated proteinase and of the oligopeptide transport system were observed. Also, the expression of the peptidases still present in the various mutants was not detectably affected. Thus, the lower growth rates can directly be attributed to the inability of the mutants to degrade casein-derived peptides. These results supply the first direct evidence for the functioning of lactococcal peptidases in the degradation of milk proteins. Furthermore, the study provides critical information about the relative importance of the peptidases for growth in milk, the order of events in the proteolytic pathway, and the regulation of its individual components.

Since milk contains only limited amounts of free amino acids and small peptides, the multiple-amino-acid auxotroph *Lactococcus lactis* is dependent on the degradation of casein for growth in this medium to high cell densities (18). The proteolytic pathway of *L. lactis*, which allows the organism to utilize casein as a source of essential amino acids, has been studied in great detail at both the genetic and biochemical levels (19, 43). On the basis of subcellular localization and the function of its components, the proteolytic pathway can be subdivided into (i) a cell envelope-associated proteinase (PrtP) which degrades casein into oligopeptides; (ii) separate transport systems for oligopeptides (Opp), di- and tripeptides (DtpT and DtpP), and amino acids (≥10 systems); and (iii) a multitude of intracellular peptidases which degrade peptides into amino acids. Both PrtP and Opp have been shown to be essential for growth in milk, whereas DtpT is dispensable (for reviews, see references 19 and 43; 12, 18). In contrast to the wealth of knowledge about the first steps of the proteolytic pathway, only a little is known about the roles of the peptidases in the degradation of casein-derived peptides.

The genes of 11 peptidases of *L. lactis*—i.e., PepO, PepO2, PepF, PepF2, PepN, PepC, PepT, PepV, PepA, PepX, and PCP—have been cloned and sequenced (most of them are reviewed in references 19 and 43; 11, 14, 16, 39, 40a). Mutants lacking either PepX, PepO, PepT, PepN, PepC, PepF, or PepA have been constructed by a gene disruption method and analyzed for their capacity to grow in milk. With the possible exception of the PepA-deficient mutant, no differences in growth rates and final cell densities between mutant and wild-type strains could be detected (8, 9, 16, 33, 36, 37, 39). These observations suggest that the functions of individual peptidases can be taken over by other peptidases with overlapping specificities or that they are not involved in casein breakdown at all.

The specificity of peptidases, determined in vitro, has been used to speculate about the roles of specific peptidases in the degradation of casein-derived peptides. For instance, PepX was proposed to be critical for the release of essential amino acids present N terminally of proline in the sequence of casein; 17% of the residues in β-casein are Pro (4, 45).

To understand the roles of individual peptidases and their possible cooperation in the degradation of casein-derived peptides, mutants with single and multiple deletions of the peptidase genes *pepN*, *pepC*, *pepX*, *pepT*, and *pepO* were constructed. For this purpose, a targeted gene inactivation method was used which allows construction of chromosomal deletions without leaving behind marker or vector DNA sequences (32). These five peptidase genes were the first to be sequenced and therefore the first to be available for inactivation. On the basis of the phenotypes of the various mutants, a simple model for the breakdown of casein-derived peptides in *L. lactis* is presented.

MATERIALS AND METHODS

Strains, plasmids, media, and growth experiments. The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* cells were grown in TY medium (2) at 37°C with vigorous agitation or on TY medium solidified with 1.5% agar, containing 100 μg of erythromycin per ml, 50 μg of kanamycin per ml, and/or 0.008% X-Gal (5-bromo-4-chloro-3-indolyl-β-galactopyranoside) when required. *L. lactis* was grown in M17 medium (49) at 30°C or on M17 medium solidified with 1.5% agar. Glucose (0.5% [GM17]) or lactose (0.5% [LM17]) was added as a carbon source. Erythromycin and X-Gal were added to 5 μg/ml and 0.008%, respectively, when needed.

Experiments with growth in milk were carried out with 10% reconstituted skim milk (Oxoid, Ltd., London, United Kingdom) steamed for 20 min on two suc-

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Name in this work	Reference
Strains			
L. lactis subsp. *cremoris*			
MG1363	Plasmid-free derivative of NCDO712		13
MG1363(pLP712)	Prt^+ Lac^+	Wild type	13
GL291(pLP712)	MG1363 Δ*pepX*	$[X]^-$	29
IM2(pLP712)	MG1363 Δ*pepO*	$[O]^-$	This work
IM3(pLP712)	MG1363 Δ*pepT*	$[T]^-$	36
IM4(pLP712)	MG1363 Δ*pepC*	$[C]^-$	This work
IM5(pLP712)	MG1363 Δ*pepN*	$[N]^-$	This work
IM6(pLP712)	MG1363 Δ*pepX*Δ*pepT*	$[XT]^-$	This work
IM7(pLP712)	MG1363 Δ*pepX*Δ*pepN*	$[XN]^-$	This work
IM8(pLP712)	MG1363 Δ*pepX*Δ*pepO*	$[XO]^-$	This work
IM9(pLP712)	MG1363 Δ*pepC*Δ*pepN*	$[CN]^-$	This work
IM10(pLP712)	MG1363 Δ*pepO*Δ*pepN*	$[ON]^-$	This work
IM11(pLP712)	MG1363 Δ*pepX*Δ*pepT*Δ*pepO*	$[XTO]^-$	This work
IM12(pLP712)	MG1363 Δ*pepX*Δ*pepT*Δ*pepN*	$[XTN]^-$	This work
IM13(pLP712)	MG1363 Δ*pepX*Δ*pepT*Δ*pepO*Δ*pepC*	$[XTOC]^-$	This work
IM14(pLP712)	MG1363 Δ*pepX*Δ*pepT*Δ*pepO*Δ*pepN*	$[XTON]^-$	This work
IM15(pLP712)	MG1363 Δ*pepX*Δ*pepT*Δ*pepN*Δ*pepC*	$[XTNC]^-$	This work
IM16(pLP712)	MG1363 Δ*pepX*Δ*pepT*Δ*pepO*Δ*pepC*Δ*pepN*	$[XTOCN]^-$	This work
IM16(pLP712, pNZ1120)	Complementation of the PepN defect by expression of *pepN* from the plasmid pNZ1120		This work
E. coli			
EC1000	MC1000 containing a copy of the *repA* gene of pWV01 in its chromosome; host strain for the pORI derivatives		27
CM89	*leu-9* Δ(*pro-lac*) *met thyA pepN102 pepA11 pepB1 pepQ10*		38
Plasmids			
pINTO3	Deletion vector for *pepO*		This work
pINTC3	Deletion vector for *pepC*		This work
pINTN3	Deletion vector for *pepN*		This work
pORI280-pepX	Deletion vector for *pepX*		29
pLP712	Prt^+ Lac^+, 54-kb proteinase/lactose plasmid of NCDO712		13
pNZ1120	Overexpression of *pepN*		51
pUK21	α-Complementation, Km^r		53

cessive days. One milliliter of an overnight culture in LM17 was washed twice with 0.9% NaCl solution and inoculated to 1% into reconstituted skim milk and incubated at 30°C. Optical densities at 600 nm and the pHs of the cultures were determined at appropriate time intervals (36). Each growth experiment was carried out in duplicate, and some of the strains were examined several times. The growth rate (μ) was determined from A_{600}s above 0.1 until the pH dropped to 5.3, when the growth of the cultures starts to slow down. The least-squares method was used for calculation of μ.

Peptidase and proteinase activity assays. Peptidase activities were determined with toluene-permeabilized cells with Lys–*p*-nitroanilide (NA) (Sigma Chemical Company, St. Louis, Mo.) and Ala–Pro–*p*-NA (Bachem Feinchemicalien AG, Bubendorf, Switzerland) as chromogenic substrates for PepN and PepX, respectively. Briefly, 1 ml of an overnight culture was washed with 20 mM Tris (pH 7.4) and centrifuged, and the resulting pellet was resuspended in 20 μl of the same buffer. Two microliters of toluene was added, and the suspension was incubated for 10 min at room temperature. Subsequently, 300 μl of 2 mM *p*-NA substrate in 20 mM Tris (pH 7.4) was added, and color development was monitored spectrophotometrically at 405 nm. Proteinase activity was determined with the chromogenic peptide S2586 (methoxy-Suc–Arg–Pro–Tyr–*p*-NA; Chromogenix AB, Mölndal, Sweden) according to the method of Exterkate (10), with the following modifications. Cells of a 1-ml overnight culture in LM17 or GM17 were washed with assay buffer (80 mM Tris [pH 7.0] containing 5 mM $CaCl_2$). The resulting pellet was resuspended in 300 μl of the same buffer containing 2 mM S2586. Prt^+ cells produce a yellow color in the reaction mixture, whereas Prt^- cells remain white. Because the peptidase mutants grew with different growth rates, inoculum series were made with dilutions of the precultures to obtain cells in the same growth phase. After a given time, cultures which had reached the same pH were selected. Aliquots of 5 ml were mixed with 0.6 ml of 15% Na_3 citrate and held on ice for 15 min to allow clearing of the milk. Cells were sedimented at 5,000 × *g* for 15 min at 4°C and washed twice with ice-cold 100 mM Tris–1% Na_3 citrate (pH 7.0) to remove traces of milk proteins. Finally, the cells were washed twice in assay buffer and the suspension was adjusted to an A_{600} of 0.2. Aliquots of 175 μl were mixed with 25 μl of 2 mM S2586 in a microtiter plate well and then covered with 50 μl of mineral oil (Wacker Chemie, Krommenie, The Netherlands [density, 1.01 g/ml]), and the increase in A_{405} was recorded with a THERMOmax microtiter plate reader (Molecular Devices Corporation, Menlo Oaks, Calif.).

Molecular cloning techniques. Molecular cloning techniques were performed essentially as described by Sambrook et al. (44). Plasmid and chromosomal DNA from *L. lactis* was isolated by the methods of Leenhouts et al. (30, 31). The 54-kb plasmid pLP712 (13) was isolated from *L. lactis* by the method of Anderson and McKay (1) and further purified by CsCl-gradient ultracentrifugation and dialysis (44). *E. coli* and *L. lactis* were transformed by electroporation as described by Zabarovsky and Winberg (56) and Holo and Nes (15), respectively.

The peptidase genes for the construction of the deletion vectors were obtained from the following sources. *pepO*, *pepT*, and *pepX* were cloned and sequenced in our laboratory (34, 36, 37). By using the nucleotide sequence from Tan et al. (48), *pepN* was amplified by PCR with VENT-polymerase (New England Biolabs, Beverly, Mass.) with primers NA (5′ CTA G**TC TAG A**CT GAA TAT TTA GGA GAA G 3′ [*Xba*I site in boldface]) and NB (5′ TAC GC**G TCG AC**A AAT TAC AAT TTT TCA GC 3′ [*Sal*I site in boldface]) and ligated in the *E. coli* vector pUK21. The PCR conditions were 4 min of denaturation, followed by 35 cycles of 1 min at 90°C, 1 min at 50°C, and 2 min 40 s at 73°C. The *pepC* gene was isolated from a *L. lactis* MG1363 chromosomal DNA bank (36) by complementation of the multiple-peptidase-deficient *E. coli* strain CM89 (38), with Ala–β-NA to detect aminopeptidase activity in the colonies. One colony harboring *pepC* was identified by PCR (nucleotide sequence data from reference 6) and Western blotting (immunoblotting) with monoclonal PepC-specific antibodies (antibodies from Chapot-Chartier et al. [5]) (35).

Construction of peptidase deletion mutants. Sixteen single- and multiple-isogenic-peptidase mutants (Table 1) were constructed from MG1363 by the method of Leenhouts and Venema (32). Gene replacement vectors were constructed for *pepN*, *pepC*, and *pepO* by using the replication-deficient plasmid pORI280 (32). Figure 1 outlines the chromosomal fragments which were used for the construction of the deletion vectors; the plasmid maps of the final constructs are also shown. The deletion vectors for *pepT* and *pepX* have been described elsewhere (29, 36). The mutant strains were analyzed by Southern hybridization for the expected chromosomal structure after the first and second

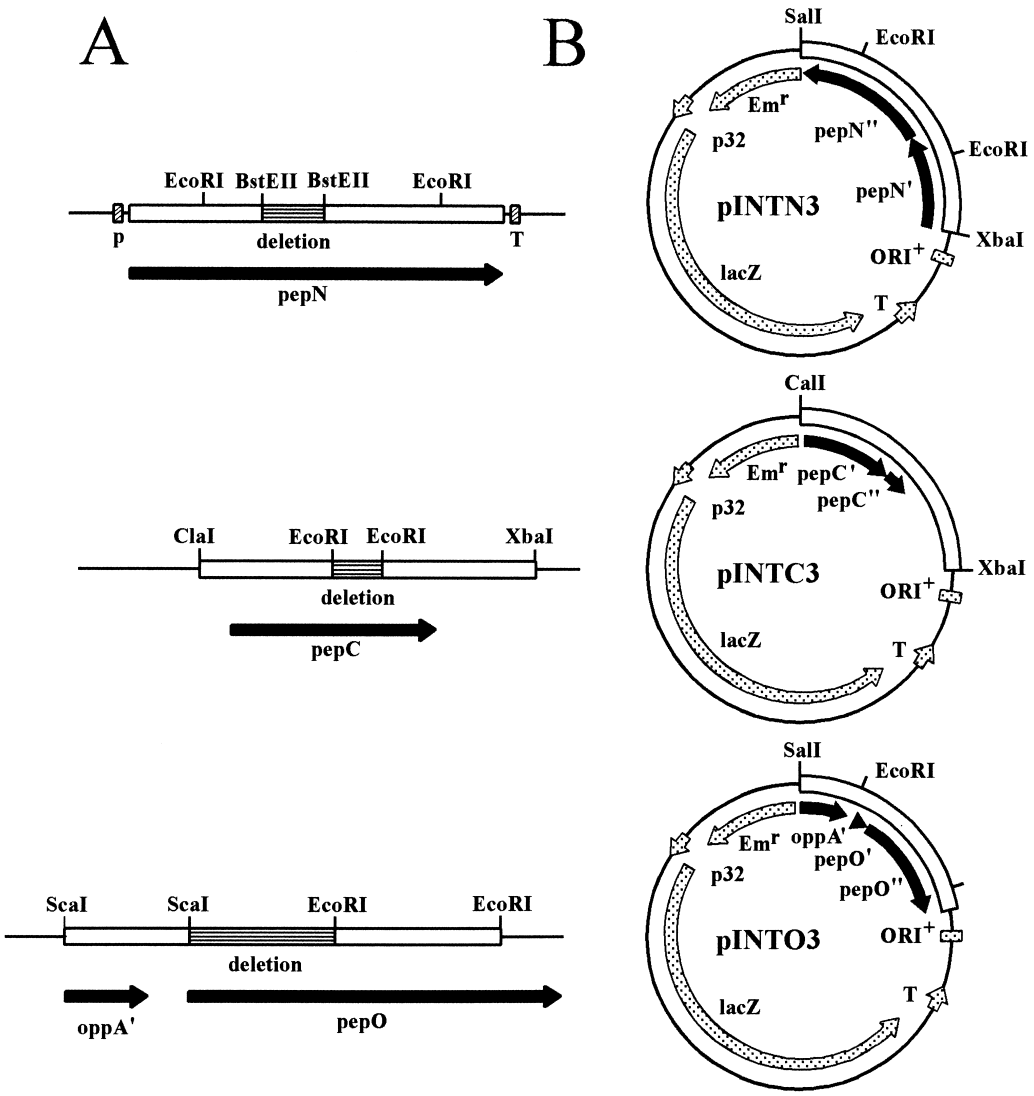


FIG. 1. Restriction maps of the peptidase genes *pepN*, *pepC*, and *pepO* (A) and the corresponding replacement vectors (B). For construction of pINTN3, a *pepN* fragment was amplified by PCR (see Materials and Methods) and subcloned with the restriction sites *Xba*I and *Sal*I, which were generated during PCR. Open bar, chromosomal region used to construct the deletion vectors; striped region, deleted part of the gene; solid arrows, open reading frames present on the corresponding DNA; P, promoter; T, terminator; stippled arrows, plasmid-encoded genes and functional areas; ORI^+, origin of replication; Em^r, erythromycin resistance gene; *lacZ*, *E. coli* β-galactosidase gene; p32, lactococcal promoter.

crossover events, respectively. The deletions in the peptidase genes resulted in the following truncated and inactive gene products: PepN, 310 amino acids (aa); PepC, 244 aa; PepO, 63 aa; PepX, 722 aa; and PepT, 233 aa. Additionally, the absence of the corresponding peptidase activities was checked with Lys–*p*-NA (PepN) or Ala–Pro–*p*-NA (PepX) as the substrate. The *pepO*, *pepT*, and *pepC* deletions were verified by Western blot analysis with the appropriate antibodies.

The proteinase-lactose plasmid pLP712 was introduced into MG1363 and its peptidase mutants by electroporation. Colonies containing pLP712 were identified on LM17 agar plates containing 0.004% of the indicator dye bromocresol purple. Yellow Lac^+ colonies were purified and checked for proteinase production with the chromogenic substrate S2586.

PCR and Southern and Western blot analyses. PCR was carried out with the GeneAmp 2400 system (Perkin-Elmer Corp., Norwalk, Conn.) under the following conditions: 0.25 mM deoxynucleoside triphosphates; 0.3 U of *Taq* polymerase (HT Biotechnology, Ltd., Cambridge, United Kingdom); and denaturation for 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 45 s at 45°C, and 90 s at 72°C. Purified chromosomal DNA was used as a template.

For Southern hybridization after agarose gel electrophoresis, DNA was transferred to Qiabrane nylon plus filters (Diagen GmbH, Düsseldorf, Germany) by the protocol of Southern, as modified by Chomczynski and Quasba (7). For detection of hybridizing DNA fragments, the enhanced chemiluminescence labelling and detection system was used (Amersham International, Amersham, United Kingdom).

Cell extracts were prepared according to the method of van de Guchte et al. (52), with the following modifications. After being washed, the cells of a 50-ml overnight culture of *L. lactis* were resuspended in 1/50 volume of 20 mM Tris HCl (pH 7.0). The cells were mixed with 1.5 g of glass beads (0.1 mm in diameter; Carl Roth GmbH & Co., Karlsruhe, Germany) and disrupted in a Shake It, Baby, cell disruptor (Biospec Products, Bartlesville, Okla.) at 4°C for 10 min. Glass beads and cell debris were removed by centrifugation (10,000 × *g*). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (26). Western analyses were carried out with polyclonal antibodies directed against PepN (1:5,000) (46), PepT (1:4,000) (46), PepO (1:8,000) (46), OppA (1:10,000) (20), and PepX (1:10,000) (5) and monoclonal antibodies directed against PepC (1:2) (5) and PepV (1:2) (25) (the ratios indicate the serum dilutions used).

For quantitative analyses of the expression levels of peptidases and OppA, cell extracts were prepared by sonication from cells grown in milk (three times for 15 s each with 15-s intervals and an amplitude of 6 μm). Proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corporation, Bedford, Mass.) with a semidry blotter and visualized by the Western-Light system (Tropix, Bedford, Mass.). The purification of OppA and the raising of

TABLE 2. Primers for multiple PCR analysis of peptidase mutants

Primer	Protein or gene	Nucleotide sequence	Fragment size (bp)	Reference
pyk-1	Pyruvate kinase	GTC TCA ACT CTT GGA CC		
pyk-2		CAG TCA AAG CAA CAA TC	1,200	28
nk-1	*pepN*	CTG AAT ATT TAG GAG AAG		
nk-2		GTG AGT TAC TTC AAC GTG	1,100	48
xk-1	*pepX*	CAA TGA CAA ATC GTT GGC		
xk-2		CCT TCT GGC AGA GCT TTC	1,000	40
ok-1	*pepO*	GAA GAA AAT TGG TCA CTC		
ok-2		AAA TCA ACG TCT TTC TCA	900	37
tk-1	*pepT*	GCA GAA ATA TGA TGA TGC		
tk-2		GCT GTA CCA GGG TGA ACG	800	36
ck-1	*pepC*	GCT TAT ACT TTC TTC TG		
ck-2		CGG TTT GAT TCT TGA CC	700	6

antibodies will be described elsewhere (20). The signal on the exposed films was quantified by scanning with the LKB 2400 GelScan XL (Pharmacia LKB Biotechnology, Uppsala, Sweden).

Intracellular amino acid and peptide pools and peptide transport assays. Strains were grown in milk and harvested at an A_{600} of 0.35 to 0.7. Cells were harvested from citrate-cleared milk as described above, washed with 80 mM Tris-HCl–1% Na_3 citrate (pH 7.5), and concentrated, and samples (1 ml each [$A_{600} \pm 15$]) were taken for analysis of the intracellular amino acid and peptide pools as described by Kunji et al. (23), with some modifications. Briefly, cells were collected by filtration on 0.45-μm-pore-diameter cellulose acetate (Schleicher & Schuell, Inc.) and washed three times with ice-cold 100 mM potassium phosphate (pH 6.5). The cells were extracted with 5% (vol/vol) perchloric acid. The extract was adjusted to pH 9.5 with 1 M KOH-$KHCO_3$, and the amino acids and peptides were dansylated in the presence of 0.5% (wt/vol) dansylchloride. The derivatized amino acids and peptides were separated by reverse-phase high-performance liquid chromatography (HPLC) with a Waters C_{18} Novapack column (150 by 4 mm; 4-μm particle size [Millipore Corp.]). Amino acids and peptides were eluted at 30°C with a linear gradient (0 to 60%) of solvent A (0.2% acetate [vol/vol] in MilliQ water; Millipore Corp.) and solvent B (100% acetonitrile); the solvents were under helium pressure, and the run time was 40 min. The flow rate was 1 ml/min, and the derivatized amino acids and peptides were detected at 254 nm. Underivatized peptides were detected at 214 nm.

For peptide transport assays, the citrate-cleared cells were washed twice with 100 mM potassium phosphate (pH 6.5). To inhibit protein synthesis, chloramphenicol (50 μg/ml) was present in all further steps. Cells ($A_{600} \pm 25$) were deenergized with 10 mM 2-deoxyglucose for 20 min at 30°C, washed twice with 100 mM potassium phosphate (pH 6.5), and resuspended in the same buffer. Prior to the transport assays, the cells ($A_{600} \pm 15$) were preincubated for 3 min in the presence of 25 mM glucose, after which 0.5 mM Gly-Leu-Gly-Leu (Bachem Feinchemikalien AG) was added. Transport was monitored by determining the intracellular amino acid and peptide pools at various time intervals as described above.

RESULTS

Construction and identification of peptidase mutants. Peptidase deletion mutants were constructed without leaving behind marker or vector DNA sequences (32), which allowed the generation of multiple mutations in subsequent rounds of mutagenesis. Because the sizes of the two portions flanking the intended deletion determine the efficiencies of the recombination events, these fragments should not be too small. When fragments with sizes of 400 and 500 bp were used in a *pepN* deletion vector, Campbell-type integrations were not obtained at the proper position. With *pepN* fragments with sizes of 1.2 and 0.9 kb, both Campbell-type integration and the subsequent excision event were easily accomplished. In agreement with these observations, the efficiency of the second recombination event of the different constructs decreased with decreasing fragment sizes: pINTN3 (1.2- and 0.9-kb fragments) > pINTC3 (0.78- and 0.9-kb fragments) > pINTO3 (0.6- and 0.88-kb fragments) (fragments left and right of the deletion are in parentheses). The difference in recombination frequency was about

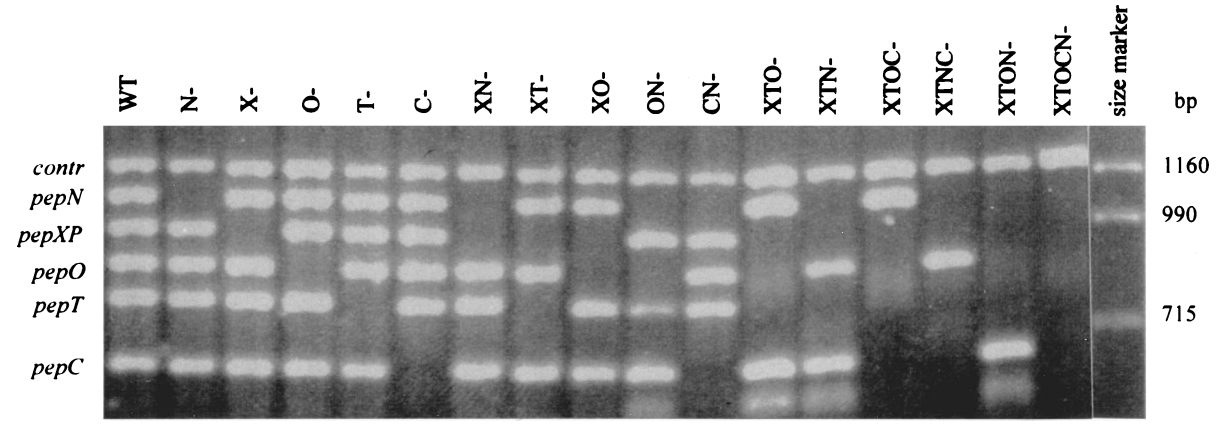


FIG. 2. PCR analysis of MG1363 and 16 peptidase-deficient mutants. Agarose (1.5% [wt/vol]) gel electrophoresis of PCR products obtained with a mixture of the 12 primers (Table 2) with chromosomal DNA of wild-type (WT) and peptidase-deficient mutants (indicated along the top of the figure). The size standard was Spp1 DNA cut with *Eco*RI. Each peptidase gene is represented by a specific band, as indicated on the left. *contr*, pyruvate kinase gene PCR product.

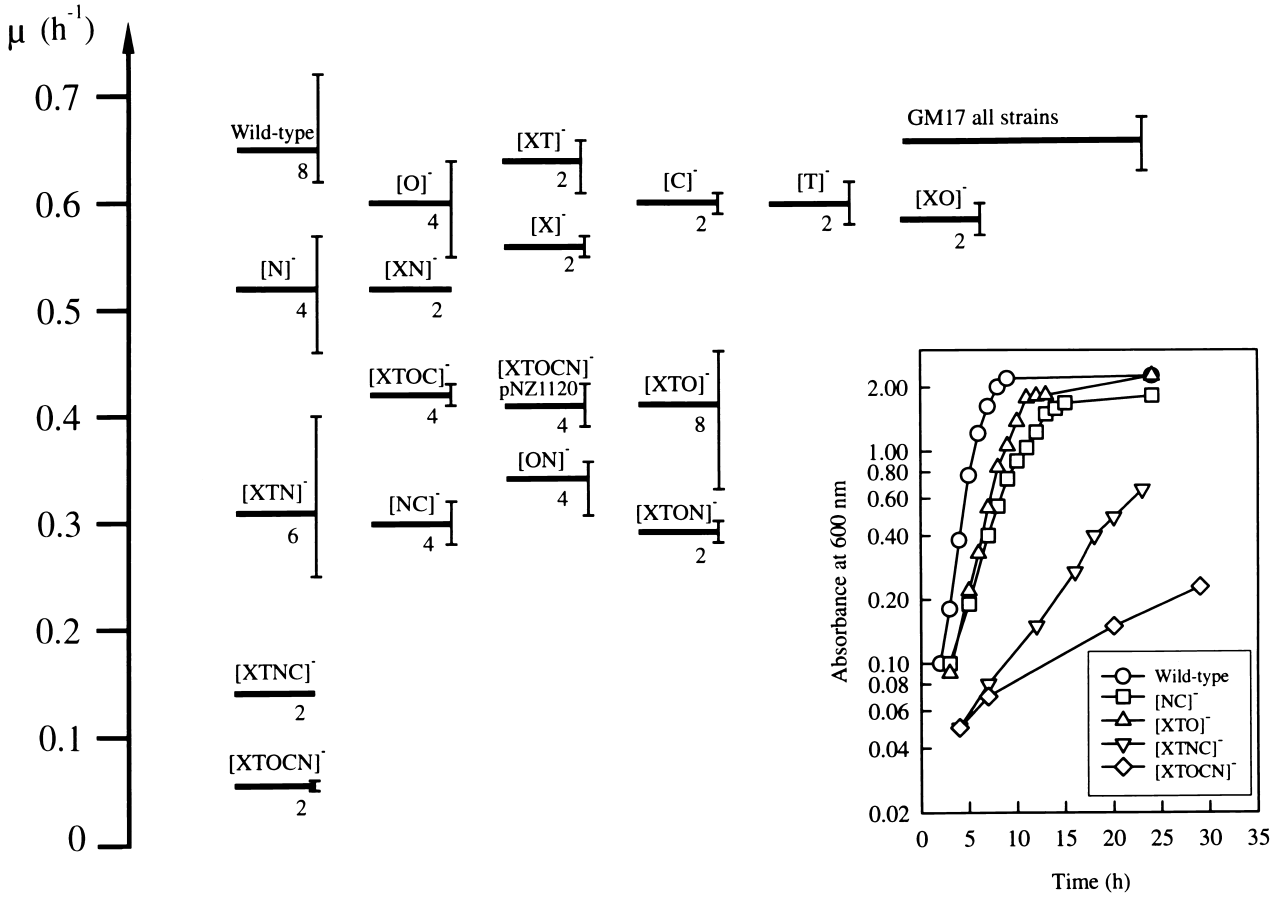


FIG. 3. Growth rates of MG1363 and peptidase-deficient mutants in milk and GM17 medium. Horizontal bars indicate the growth rate. Vertical bars mark the variation of the data by showing the range of the highest and the lowest values measured for a particular strain. Numbers under the horizontal bar indicate the number of independent measurements carried out.

10-fold between pINTN3 and pINTO3. Similar observations were made by Biswas et al. (3). Biswas et al. were still able to obtain Campbell-type integrations with fragments as small as 300 bp. In our experience, a minimal size of the deletion-flanking fragments of 600 bp was required for a detectable frequency of recombination (as determined from the excision event: 10^{-4} ml^{-1} after 30 generations of growth without antibiotic pressure).

A set of 12 primers (Table 2) was designed with which the correct chromosomal structure of the mutant strains could be assessed in a single PCR. For each peptidase gene, specific primer couples were made: one primer complementary to a region which is present in both the wild-type and mutant chromosomes and a second primer complementary to the part which is deleted in the mutants. Thus, subjecting a particular mutant to PCR should result in the absence of the corresponding PCR product. As a positive control for the PCR, two primers for the pyruvate kinase gene were included (28). For easy analysis, the primers were designed in such a way that the various amplified products would differ by about 100 bp. Figure 2 shows the products of the PCRs performed with chromosomal DNAs of all 16 peptidase-negative mutants. Each mutant showed the expected spectrum of bands, confirming that all had the designated genotype.

Growth of peptidase mutants in milk. Growth and acid production in milk of all peptidase mutants were monitored until the cultures reached the stationary phase. The results are summarized in Fig. 3. The growth curves of some of the mutants are shown in the inset of the figure. The growth rates in GM17 medium are included for comparison and were similar for the wild type and all mutants. Remarkably, even the absence of five peptidases had no influence on the growth of *L. lactis* in this complex medium.

As was observed before (9, 33, 36, 37), single deficiencies in PepX, PepO, PepC, and PepT had no significant effect on the growth rates of the mutants in milk, with the possible exception of the strain lacking PepN. This strain grew slightly, but significantly, more slowly than the wild type. The general trend in the growth rates of the mutants in milk was that the more peptidases were lacking, the slower the strain grew. Ultimately, the mutant with a fivefold deficiency in peptidases PepX, PepT, PepO, PepC, and PepN ($[XTOCN]^-$) had a growth rate of 0.055, which is more than 10 times lower than that of the wild type. In addition to the effect of the mutations on the growth rates, some of the strains showed a prolonged lag phase. This was most pronounced for the $[XN]^-$, $[XTN]^-$, and $[XTON]^-$ mutants.

To rule out that the low growth rate of the $[XTOCN]^-$ mutant was caused by a side effect of the mutation procedure, the PepN expression plasmid pNZ1120 (51) was introduced into this strain. The $[XTOCN]^-$(pNZ1120) strain had the same growth rate in milk as the parental $[XTOC]^-$ strain, indicating that the drastic difference in growth rates between

TABLE 3. PrtP activity in wild-type and peptidase-deficient mutant strains

Strain[a]	Relative PrtP activity (%)[b]
Wild type	100
[C]$^-$	100
[X]$^-$	113
[N]$^-$	111
[ON]$^-$	112
[CN]$^-$	96
[XTO]$^-$	112
[XTN]$^-$	111
[XTOC]$^-$	110
[XTON]$^-$	118
[XTNC]$^-$	98
[XTOCN]$^-$	82
[XTOCN]$^-$ (pNZ1120)	89

[a] Strain designations are given according to Table 1. All strains contained the lactose and proteinase plasmid pLP712.

[b] Cells were harvested during exponential growth in milk at pH 5.5. PrtP activity was measured as described in Materials and Methods; the 100% value corresponds to 6 nmol min^{-1} mg of protein^{-1}.

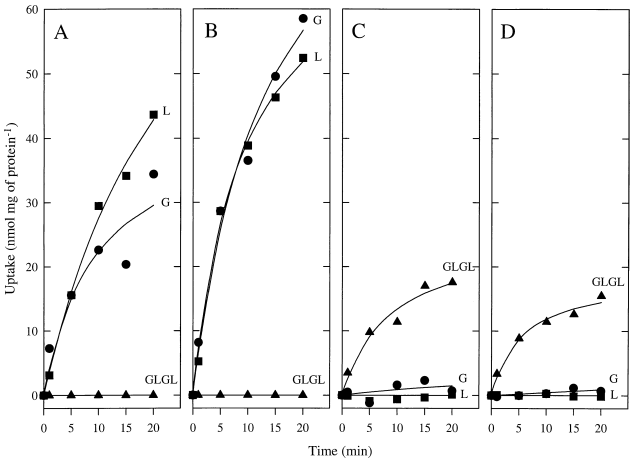


FIG. 4. Uptake of Gly-Leu-Gly-Leu by the oligopeptide transport system of the wild type (A) and the [XTOC]$^-$ (B), [XTNC]$^-$ (C), and [XTOCN]$^-$ (D) mutants. Cells were harvested during exponential growth in milk. Prior to the transport assays, cells were deenergized as described in Materials and Methods. Cell suspensions were preenergized for 3 min with 0.5% (wt/vol) glucose in 100 mM potassium phosphate (pH 6.5) before the uptake was started by addition of 0.5 mM Gly-Leu-Gly-Leu (GLGL). Peptide and amino acid pools were determined as described in Materials and Methods. Amino acids are indicated by their one-letter denomination.

[XTOC]$^-$ and [XTOCN]$^-$ is exclusively caused by the absence of PepN in the latter strain.

To investigate whether the decreased growth rates in milk were due to the absence of particular peptidases and not to lower levels of expression or activity of essential components of the proteolytic pathway, PrtP and Opp activities in the various peptidase mutants were examined.

Activity of PrtP in peptidase mutants during growth in milk. The specific PrtP activity of MG1363(pLP712) (wild type) was measured during growth in milk from an A_{600} of 0.2 to an A_{600} of 1.2 (pH 6.2 to 5.2). This activity varied less than 10% within the range of cell densities assayed (data not shown), indicating the lack of growth-related regulation in this range. PrtP activity in a representative selection of mutants was measured at different cell densities. Table 3 shows the results for samples which were taken when the cultures were in the logarithmic growth phase (around pH 5.5), i.e., when amino acids essential for growth can be obtained only from casein-derived peptides (18). The specific activities of PrtP were similar in all mutant strains, indicating that the lower growth rates observed were not caused by the reduced activity of PrtP.

Activity of Opp in peptidase mutants. A second possibility for the lower growth rates of the mutants in milk could be decreased expression of the genes specifying the oligopeptide transport system or decreased activity of this system as a result of the possible intracellular accumulation of peptides due to the absence of certain peptidases. Therefore, we have measured the expression of OppA, the membrane-anchored binding protein of the system, as well as the Opp transport activity in selected strains grown in milk.

The relative amounts of OppA present in the mutants and the wild-type strain were determined by Western blotting and appeared to be comparable (Table 4). The uptake of the Opp substrate Gly-Leu-Gly-Leu (50) in the wild-type strain and in the [XTOC]$^-$, [XTNC]$^-$, and [XTOCN]$^-$ mutants was measured. In the former two strains, the tetrapeptide was completely degraded to glycine and leucine upon entry into the cells (Fig. 4A and B). In contrast, in the [XTNC]$^-$ and the [XTOCN]$^-$ mutants, Gly-Leu-Gly-Leu accumulated intracel-

TABLE 4. Relative levels of expression of peptidases and OppA in the wild-type strain MG1363 and peptidase-deficient mutants

Strain	Relative expression (%) of[a]:					OppA
	PepN	PepC	PepT	PepV	PepX	
MG1363	100 ± 17	100 ± 15	100 ± 20	100 ± 20	100 ± 16	100 ± 12
[C]$^-$	—[b]	—	—	—	90 ± 16	—
[N]$^-$	—	—	—	—	100 ± 12	—
[XT]$^-$	110 ± 16	—	—	—	—	—
[XN]$^-$	—	—	90 ± 13	—	—	—
[XO]$^-$	100 ± 18	—	80 ± 15	—	—	—
[CN]$^-$	—	—	90 ± 25	—	120 ± 18	—
[ON]$^-$	—	—	90 ± 20	—	150 ± 16	—
[XTO]$^-$	100 ± 18	—	—	—	—	—
[XTN]$^-$	—	90 ± 4	—	70 ± 38	—	160 ± 22
[XTOC]$^-$	110 ± 23	—	—	—	—	—
[XTON]$^-$	—	130 ± 12	—	90 ± 16	—	190 ± 28
[XTNC]$^-$	—	—	—	70 ± 16	—	170 ± 15
[XTOCN]$^-$	—	—	—	90 ± 31	—	140 ± 32

[a] The accuracy of the data is indicated by the relative 95% confidence interval of the population mean. Except for PepC (n = 6), n = 4.

[b] —, not determined.

lularly, while Leu and Gly pools did not increase significantly (Fig. 4C and D). Apparently, these mutants were unable to degrade the internalized Gly-Leu-Gly-Leu. When one molecule of Gly-Leu-Gly-Leu is degraded completely, two molecules (each) of glycine and leucine are formed, and the accumulation rates of Gly and Leu should be twice as high as that of the whole peptide. Taking this into account, the calculated initial transport rates were similar for the wild type and the $[XTNC]^-$ and the $[XTOCN]^-$ mutants (1.8, 2.1, and 2.2 nmol min^{-1} mg of $protein^{-1}$, respectively), and somewhat higher for $[XTOC]^-$ (2.8 nmol min^{-1} mg of $protein^{-1}$). Thus, in agreement with the observation that the expression of OppA was not down-regulated as a result of the peptidase mutations, the transport activity was not altered significantly.

Expression of peptidases in peptidase mutants. Because nothing is known about the influence of peptidase gene deletions on the expression of other peptidases in the cell, we quantitated the levels of PepN, PepC, PepT, PepV, and PepX in the various peptidase mutants by an immunological approach. PepO cannot be measured in this way, since another protein of approximately the same size reacts with the PepO-specific antibodies (37). Table 4 presents the relative expression levels of the different peptidases in a selected set of strains. The amounts of peptidases which were expressed in the wild-type and mutant strains were found to be very similar, indicating that expression of these peptidases was not influenced by the presence or absence of other peptidases.

Amino acid and peptide pools in the wild type and in multiple-peptidase mutants. The results presented so far show that inactivation of peptidases does not reduce the level of expression or activity of the other components of the proteolytic system. This supports the suggestion that the decreased growth rates of peptidase mutants of *L. lactis* in milk are directly caused by the inability to efficiently break down casein-derived peptides to the amino acids needed for growth.

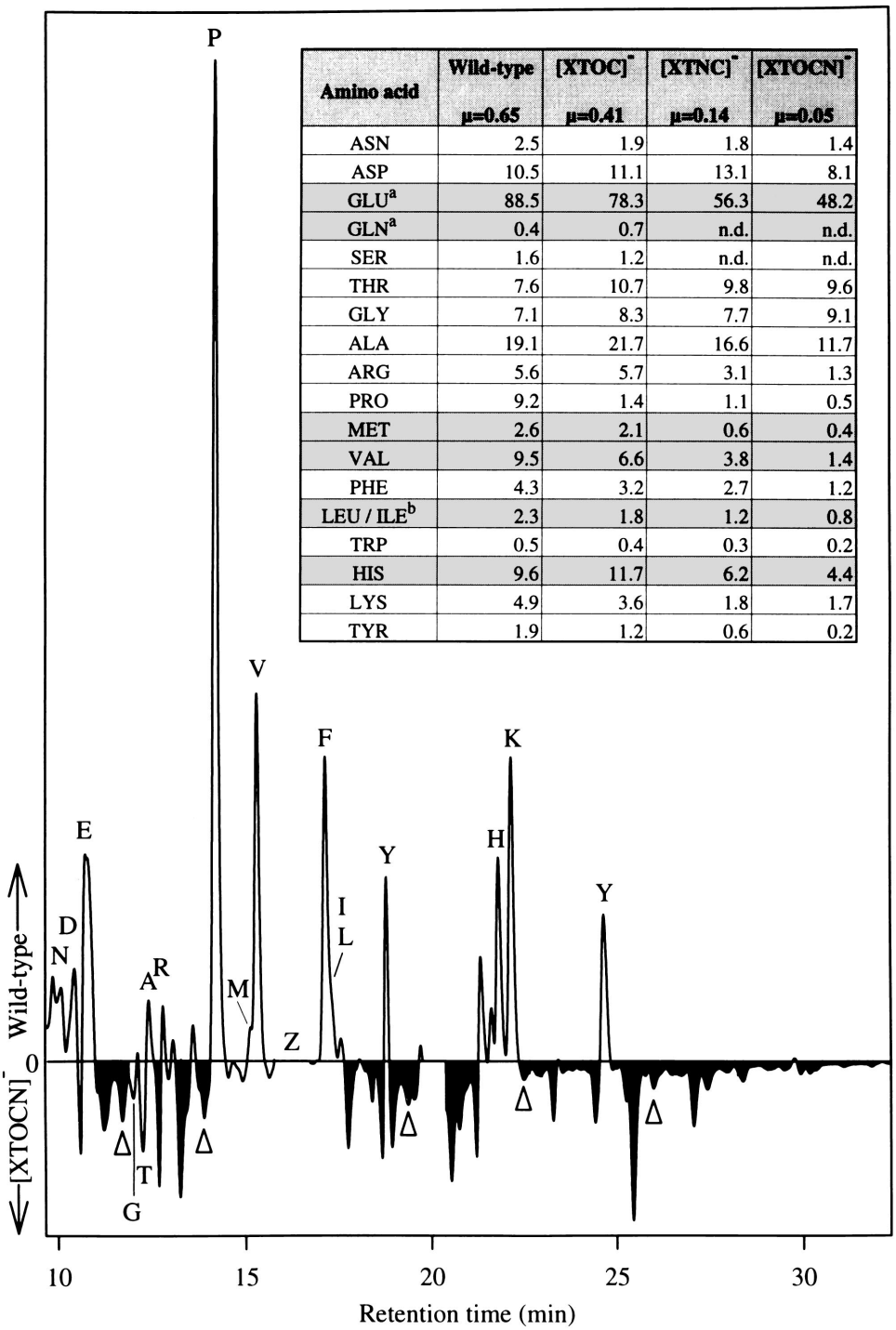
To substantiate this notion, the amino acid composition and peptide composition of the intracellular fractions of cells harvested during exponential growth in milk were examined. Figure 5 shows the difference HPLC chromatogram of dansylated intracellular pools of amino acids and peptides of the wild-type minus those of the $[XTOCN]^-$ strain. Positive peaks represent compounds present in larger amounts in the wild-type strain, while negative peaks represent substances more abundant in the mutant. Interestingly, almost all peaks in the HPLC chromatogram of the wild type could be attributed to amino acids on the basis of retention times (positive peaks in Fig. 5), while the intracellular fraction of the $[XTOCN]^-$ strain showed, in addition to amino acids (see inset in Fig. 5), a multitude of other peaks (negative peaks colored black in Fig. 5). Because the compounds in question can be dansylated and absorb at 214 nm, they most likely correspond to peptides. With the exception of five additional peptide peaks (triangles in Fig. 5), all peaks found in the chromatogram of the $[XTOC]^-$ strain were also present in the chromatogram of the wild type. The $[XTNC]^-$ chromatogram was almost identical to that of $[XTOCN]^-$, although the relative amounts of the compounds varied somewhat (data not shown). The pools of Glu, Arg, Met, Val, Phe, Leu/Ile, Trp, His, Lys, and Tyr were considerably lower in the $[XTOCN]^-$ and $[XTNC]^-$ strains than in the $[XTOC]^-$ and wild-type strains (Fig. 5, inset). Strikingly, the proline pools in these multiple-peptidase mutants were dramatically lower than those in the wild-type strain. Other amino acid pools were unaffected or slightly increased in the cases of the $[XTOCN]^-$ and $[XTNC]^-$ mutants. These results show that the observed lower growth rates of the $[XTOCN]^-$ and $[XTNC]^-$ mutants are indeed related to a severely impaired ability to degrade peptides and to release essential amino acids.

DISCUSSION

The proteolytic pathway, which allows *L. lactis* to liberate and utilize amino acids from casein, consists of a cell envelope-associated proteinase, transport systems for peptides and amino acids, and peptidases. The crucial role of both the proteinase and the oligopeptide transport system is well established (19, 43). In the present study, we have investigated the function of peptidases in the degradation of casein-derived peptides. Up to five peptidase genes (*pepN*, *pepC*, *pepX*, *pepT*, and *pepO*) could be inactivated without impairing growth in a complex medium like M17. In contrast, deletion of increasing numbers of these peptidase genes led to decreasing growth rates in milk. A strain lacking all five peptidases grew more than 10 times more slowly than the wild type. That this phenotype is caused by a decrease in expression or activity of the other components of the proteolytic pathway has been ruled out; i.e., neither PrtP or Opp nor other peptidase activities were seriously affected by the peptidase mutations. We therefore conclude that the decreased growth rates were the direct result of the reduced capacity of the mutant cells to break down peptides to free amino acids needed for growth. This conclusion is supported by several observations. First, in contrast to the wild-type strain, the $[XTNC]^-$ and $[XTOCN]^-$ mutants accumulated peptides intracellularly. This is in agreement with the observation that the peptide Gly-Leu-Gly-Leu is not degraded and, therefore, accumulated in cells in the absence of PepX, PepT, PepN, and PepC. In another study, we will present further evidence for the intracellular accumulation of other peptides (22). Second, in the $[XTNC]^-$ and the $[XTOCN]^-$ mutants, the intracellular amino acid pools were significantly lower than those in the wild-type and $[XTOC]^-$ strains. This is striking in view of the fact that fast-growing cells consume amino acids at a higher rate than slowly growing ones, or, stated differently, that slowly growing cells have more time to accumulate amino acids.

Most peptidases of the proteolytic system of *L. lactis* have now been characterized at the enzyme and DNA levels, providing information, among others, about the class of enzymes, substrate specificities, and sequence similarities. The results presented here allow, for the first time, evaluation of the physiological roles of five of these peptidases in the growth of the organism in milk. With the possible exception of PepX, each of the five peptidases contributes significantly to the delivery of amino acids from casein-derived peptides. Certain peptidases seem to play a more prominent role than others. For instance, inactivation of PepN alone leads to a significant decrease in the growth rate. Apparently, no other peptidase or combination of peptidases can fully take over the function of PepN. Also, combinations of mutations including *pepN* have more drastic effects on growth rates in milk than the same number of mutations without *pepN*; e.g., in Fig. 3 compare $[C]^-$ with $[NC]^-$, $[O]^-$ with $[ON]^-$, $[XT]^-$ with $[XTN]^-$, $[XTO]^-$ with $[XTON]^-$, and $[XTOC]^-$ with $[XTOCN]^-$. PepN alone is not sufficient to provide cells with all of the amino acids necessary for the maximum growth rate, as was demonstrated by the fact that two multiple-peptidase mutants which still produced PepN ($[XTO]^-$ and $[XTOC]^-$) grew more slowly than the wild type.

The roles of PepX, PepO, PepC, and PepT can be evaluated only from their inactivation in combination with other peptidases, since their inactivation alone had no significant effect on growth rates. Our data indicate that the function of these enzymes can be taken over by other peptidases in the cell.



Amino acid	Wild-type μ=0.65	[XTOC]⁻ μ=0.41	[XTNC]⁻ μ=0.14	[XTOCN]⁻ μ=0.05
ASN	2.5	1.9	1.8	1.4
ASP	10.5	11.1	13.1	8.1
GLU[a]	88.5	78.3	56.3	48.2
GLN[a]	0.4	0.7	n.d.	n.d.
SER	1.6	1.2	n.d.	n.d.
THR	7.6	10.7	9.8	9.6
GLY	7.1	8.3	7.7	9.1
ALA	19.1	21.7	16.6	11.7
ARG	5.6	5.7	3.1	1.3
PRO	9.2	1.4	1.1	0.5
MET	2.6	2.1	0.6	0.4
VAL	9.5	6.6	3.8	1.4
PHE	4.3	3.2	2.7	1.2
LEU / ILE[b]	2.3	1.8	1.2	0.8
TRP	0.5	0.4	0.3	0.2
HIS	9.6	11.7	6.2	4.4
LYS	4.9	3.6	1.8	1.7
TYR	1.9	1.2	0.6	0.2

FIG. 5. Difference HPLC chromatogram of intracellular amino acid and peptide fractions of the wild type minus those of the $[XTOCN]^-$ mutant during growth in milk. Samples were taken during exponential growth in milk. Intracellular fractions were analyzed as described in Materials and Methods, and the values from the HPLC chromatogram of the $[XTOCN]^-$ mutant were then subtracted from those of the wild type. Positive peaks (white) represent compounds which are present in larger amounts in the wild type, while negative peaks (black) represent compounds which are present in larger amounts in the $[XTOCN]^-$ mutant. Amino acids are indicated at their peaks with the one-letter denomination. Triangles indicate the retention times of putative peptide peaks present in the fourfold $[XTOC]^-$ mutant. The retention time of γ-aminobutyrate is indicated by Z. The inserted table presents the intracellular amino acid concentrations of the wild type and the $[XTOC]^-$, $[XTNC]^-$, and $[XTOCN]^-$ mutants during growth in milk; the corresponding growth rates are shown in the first row. Essential amino acids are indicated within shaded bars. Footnotes: a, in the cases of Glu and Gln, either one or the other amino acid is essential. b, because of the high intracellular Phe pools, Ile and Leu are present in a shoulder peak of Phe; the values for Ile/Leu were estimated from the height of the shoulder of the Phe peak. n.d., amino acids could not be quantified because of overlapping peptide peaks.

PepC inactivation in a *pepN* background results in a drastic further decrease in the growth rate, indicating that in addition to PepN, PepC is an important enzyme for the release of amino acids. PepC is likely to have a more narrow specificity or a lower level of overall activity than PepN in *L. lactis* MG1363, because it cannot fully complement the *pepN* mutation.

A prominent role for the endopeptidase PepO is observed in a *pepN* background. In the absence of the general aminopeptidase PepN, amino acids are most likely formed by PepO-mediated degradation of oligopeptides to tri- and dipeptides, which become the substrates of the di- and tripeptidases PepV and PepT. The importance of PepO can also be seen in the differences between the growth rates of the $[XT]^-$ and $[XTO]^-$ strains and between those of the $[XTNC]^-$ and $[XTOCN]^-$ strains.

The contribution of PepT in casein degradation can be evaluated by comparing the growth rate of $[XN]^-$ with that of $[XTN]^-$ and that of $[XO]^-$ with that of $[XTO]^-$. Apparently, the PepT function cannot be fully taken over by PepN or PepC, which are both known to degrade tripeptides in vitro (41, 42, 47) and in vivo (22).

A crucial role for growth in milk of PepX has been proposed because of the high proline content of β-casein and the high frequency at which essential amino acids are found at the N-terminal side of Pro (4, 45). However, a deficiency in PepX alone had no effect on the growth rate in milk, as was observed before (33). Combination of a deficiency in PepX with any of the other peptidase mutations had not the slightest effect on growth; e.g., compare the growth rates of $[T]^-$ and $[XT]^-$, $[O]^-$ and $[XO]^-$, and $[N]^-$ and $[XN]^-$ (Fig. 3). Strikingly, the proline pools of multiple-peptidase mutants lacking PepX were 10- to 20-fold lower than that of the wild type. Clearly, the supply of proline is not a limiting factor for growth. The results obtained so far do not allow a final assessment of the role of PepX, since multiple-peptidase mutants still containing PepX have not yet been constructed.

Now that we have gained a better understanding of the roles of individual peptidases in vivo, we can hypothesize about the sequence of events that take place during utilization of milk proteins. In the first step, PrtP generates from casein 4- to 30-aa-residue peptides (17). Subsequently, peptides from this pool are translocated into the cell by the Opp system. Activity of this transport system, which has a substrate range of peptides with from 4 up to at least 8 residues (21, 50), will determine the quantity and quality of peptides available for the intracellular peptidases. These peptides are first broken down by peptidases with broad specificity, both from the N terminus (PepN and PepC) and endolytically (PepO), leading to smaller peptides as well as free amino acids. Although PepN and PepC can release amino acids directly from peptides, both general aminopeptidases alone or together are unable to supply the cell with sufficient amounts of amino acids for maximal growth rates. Apparently, a significant number of the peptides require another route of degradation. In this route, PepO is likely to be critical, because inactivation of this endopeptidase in addition to PepN leads to a severe decrease in growth rate. The final steps of peptide degradation are most likely performed by the tripeptidase PepT and the dipeptidase PepV.

The fact that the fivefold mutant had a growth rate in milk close to zero, whereas growth of the mutant in amino acid-containing media is comparable to that of the wild type, indicates that we have identified enzymes crucial in the breakdown route of casein-derived peptides. It also indicates that other peptidases with similar activities are not present in the cell. This conclusion is supported by the observation that in the fivefold mutant, peptides are indeed accumulating in the cell.

Since intracellular peptidases also have a role in the breakdown (turnover) of endogenous and misfolded proteins (54, 55), it is surprising that the fivefold mutant, albeit severely impaired in its growth in milk, grows with wild-type μ_{max} in complex broth (GM17). During growth in GM17, accumulation of intracellular peptides is not observed (data not shown). This suggests that the residual peptidolytic activity is sufficient for these housekeeping functions of the cell. It should be emphasized that such housekeeping functions may have a more prominent role under stress conditions, for instance, when cells are energy starved (24), which was not investigated in this study.

The estimation of the activities of the different components of the proteolytic system in the wild type and in the peptidase mutants provides, for the first time, important insights into the regulation of the individual components during growth in milk. Because no major changes in the activities of PrtP, Opp, and the peptidases were observed in the various peptidase backgrounds, the expression through changes in amino acid and/or peptide levels in the cell does not seem to be subject to regulation. This is striking, because one would expect that under the nitrogen starvation conditions prevailing in the multiple-peptidase mutants, mechanisms would exist to compensate for the peptidase deficiency by increasing the expression levels of other components of the proteolytic system. Our studies indicate that *L. lactis* lacks such mechanisms.

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